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Lymphoproliferative Responses to Human Herpesvirus-6 Variant A and Variant B in Healthy Adults

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Human herpesviurs-6 (HHV-6) isolates can be classified into variants A and B, and over 95% of people older than 2 years of age are seropositive for either or both variants. However, the prevalence of the two HHV-6 variants is still not defined since the serological methods used at present cannot discriminate one variant from the other. Lymphoproliferative responses to glycine extracted cellular antigens from human herpesvirus-6 (HHV-6) GS strain (variant A)- and Z 29 strain (variant B)-infected T-lymphoid cell lines were examined in healthy Swedish and Japanese adults. Nine of 36 (25%) persons had responses to the GS antigen, while 21/36 (58%) had responses to the Z 29 antigen ($P = 0.008$). Individuals with low anti–HHV-6 IgG titers ($\leq$320) were more likely to respond to the Z 29 antigen than to the GS antigen ($P = 0.006$), while there was no difference in those with high anti–HHV-6 IgG titers ($\geq$1280). Three of 7 Japanese adults had lymphoproliferative responses to the GS antigen compared with 6/29 Swedes (not significant), and 7/7 Japanese had lymphoproliferative responses to the Z 29 antigen compared with 14/29 Swedes ($P = 0.03$). Lymphoproliferative responses were neither related with the presence of HHV-6 DNA nor related with the presence of HHV-7 DNA in peripheral blood cells. These results suggest a higher prevalence of HHV-6 variant B than variant A in both Swedes and Japanese adults, and possibly a difference in either the HHV-6 virus strains and/or the nature of immune response of Swede and Japanese. ***J. Med. Virol. 57:134–139, 1999.***

KEY WORDS: anti–HHV-6 IgG; HHV-6 DNA; Swede; Japanese

INTRODUCTION

Human herpesvirus 6 (HHV-6) is a human herpesvirus discovered recently, which infects humans within the first two years of life [Okuno et al., 1989; Hall et al., 1994]. HHV-6 infection is widespread throughout the world, with rates of seroprevalence ranging from 72% to 100% in the healthy population, but prevalence of HHV-6 variants A and B in healthy adults has been studied only to a limited extent since the serologic methods used at present cannot discriminate the two variants [Braun et al., 1997]. HHV-6 viruses can be divided into variant A and variant B according to genomic, antigenic, and biological differences [Ablashi et al., 1991]. By polymerase chain reaction (PCR) methods, HHV-6 variant B has been found to be more common than variant A in peripheral blood cells (PBL) of healthy persons, but the overall rates of HHV-6 DNA detected by PCR have been less than 20% in these studies [Di Luca et al., 1994; Wang et al., 1996].

HHV-6 variant A has not been correlated with any disease. In contrast, primary infection of HHV-6 variant B has been identified as the cause of exanthem subitum, and accounts for the majority of symptomatic primary HHV-6 infections in infants [Yamanishi et al., 1988; Braun et al., 1997]. HHV-6 infection may cause encephalitis in both immunocompetent and immunocompromised individuals [Drobyski et al., 1994; Knox et al., 1995; Novoa et al., 1997]. A link between multiple sclerosis and HHV-6 is also presently discussed [Challoner et al., 1995; Enbom et al., 1997; Soldan et al., 1997]. HHV-6 inhibits the growth of stem cells in in vitro studies and HHV-6 infection is related to delayed engraftment and marrow failure after bone marrow transplantation [Knox et al., 1992; Drobyski et al.,

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1993; Flamand et al., 1995; Wang et al., 1996]. Moreover, HHV-6 infection of peripheral blood cells in vitro results in reduced interleukin-2 synthesis and inhibits cellular proliferation [Horvat et al., 1993; Flamand et al., 1995]. Progressive immunodeficiency associated with HHV-6 in an infant has also been described [Knox et al., 1995]. There is only one published study on T-cell proliferative responses to HHV-6, and only antigen from HHV-6 GS strain was used in that study [Yakushijin et al., 1991]. Due to the increased clinical importance of this virus, further studies on T-cell memory responses to HHV-6 antigens are of interest.

Human herpesvirus-7 (HHV-7) is another human herpesvirus discovered recently, and like HHV-6, it may cause exanthem subitum and CNS symptoms in pediatric patients [Ablashi et al., 1995; Torigoe et al., 1996]. Like human cytomegalovirus (CMV), both HHV-6 and HHV-7 are beta herpesviruses. Cross-reactivities among the three viruses have been shown serologically and in studies of memory T-cell clones [Linde et al., 1990; Yasukawa et al., 1993].

In this study, we examined the T-cell proliferative responses to glycine-extracted cellular antigens predominantly containing nucleocapsids of GS strain and Z 29 strain, representing HHV-6 variants A and B, respectively, in healthy adults. The results have been analyzed with anti–HHV-6 IgG titers and with the presence of HHV-6 DNA in PBL. In order to study potential cross-reactivities, CMV specific lymphoproliferative responses and plasma CMV IgG levels, as well as the presence of HHV-7 DNA in PBL, were analyzed.

MATERIALS AND METHODS

Cells and Viruses

The GS strain (variant A) of HHV-6 was grown in HSB-2 cells and the Z 29 strain (variant B) was grown in MOLT-3 cells (both viruses and cell lines were kindly donated by Professor R. Gallo). Glycine-extracted antigens, predominantly containing nucleocapsids, were prepared according to a previously described method [Kettering et al., 1977] when the rate of virus-infected cells exceeded 50%. Briefly, HHV-6–infected cells were collected by centrifugation at 1,500 rpm for 15 min, resuspended in 1/20 of the original culture volume of 0.1 M glycine buffer (pH 9.5), sonicated on ice and centrifuged at 2,000 rpm for 20 min at 4°C in a Centrikon H-401 (Kontron Instruments, Switzerland). The supernatant fluids were collected and used as antigen preparations. To control the presence of live viruses, the preparations were inoculated to HSB-2 or MOLT-3 cells. No infected cells were observed during 3 weeks after the inoculation as analyzed by an immunofluorescence method [Linde et al., 1990]. The same amount of mock-infected HSB-2 and MOLT-3 cells were prepared in the same way and used as control antigens. The protein contents of the preparations were determined by the Lowry method. Nuclear antigens mainly containing nucleocapsids of human cytomegalovirus (CMV) AD 169 and varicella zoster virus (VZV) local strain 9/84 grown in fetal fibroblast cells were used as control antigens for the specific lymphoproliferation assays [Ljungman et al., 1985]. The antigens prepared were divided into small tubes and kept at -70°C until use.

Subjects and Specimens

Thirty-six healthy adults (12 male, 24 female) with a median age of 38 years (range 24–60) were enrolled in this study. Twenty-nine (female/male: 23/6) were Swedish staff members of either the Swedish Institute for Infectious Disease Control or the Huddinge University Hospital (Stockholm, Sweden), and seven (female/male: 1/6) were Japanese scholars visiting the Karolinska Institute (Stockholm, Sweden).

Peripheral blood samples were drawn into tubes containing EDTA and heparin, respectively. Buffy coat was collected from the blood tubes with EDTA as the source of PBL. The cells were counted and frozen at -20°C until PCR analysis was performed. The plasma was also collected and kept at -20°C until serological analysis. The blood samples in the heparin tubes were used for lymphocyte proliferation assay.

Lymphocyte Proliferation Assay

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden). The cells were washed twice with RPMI 1640, then resuspended in culture medium consisting of RPMI 1640 supplemented with glutamine, penicillin, streptomycin, 10^{-5}-mol β_2-mercaptoethanol (Life Technologies, Paisley, Scotland) and 10% human AB+, CMV seronegative serum. The human serum was assessed previously for its ability to support PBMC cultures stimulated with the CMV antigen and phytohemagglutinin (PHA, Murex Diagnostics, Dartford, England). Serum of the same batch was used throughout this study. The PBMC were adjusted to 1.5×10^6 cells/ml and 100 μl of the cell suspension were added per well into 96-well flat-bottom plates (Nunclon Delta, Nunc, Aarhus, Denmark). All assays were carried out in quintuples. The CMV antigen, VZV antigen and PHA were used at the optimal doses determined previously [Ljungman et al., 1985]. The optimal doses of HHV-6 antigens for lymphocyte stimulation were chosen by titration of the antigens and their control antigens. All the antigens and PHA were diluted in the supplemented RPMI 1640, and 100 μl were added per well. Proliferation was measured by [^{3}H]-thymidine (Amersham, U.K.) incorporation (1 μCi/well, 4-hr pulse) on day 6. The cells were harvested onto glass fiber pads, and counts per minute (CPM) were measured in a 1205 Beta-Plate (LKB Wallac, Turku, Finland). Results were expressed as stimulation indexes (SI), which were derived from the CPM after antigen stimulation divided by the CPM obtained after stimulation with the corresponding control antigen. The CPM after CMV antigen or VZV antigen stimulation were divided by that of one of the HHV-6 control antigens with the highest CPM, and the CPM of PHA were divided by that of medium alone to obtain the SI. A

TABLE I. Lymphoproliferative Responses to HHV-6 Nuclear Antigens at Different Concentrations

	GS antigen (μg/ml)			Z29 antigen (μg/ml)		
	4	8	16	3	6	12
Mean net CPM[a] (×10^3, n = 4)	8	11	8	11	14	13
Range	0.2–18	3–22	2–25	3–14	3–29	4–28
Mean SI[b] (n = 4)	15	15	5	14	14	11
Range	1–46	4–36	2–17	4–20	6–22	6–30

[a]The net counts per minute (net CPM) was derived from reducing the CPM of peripheral blood mononuclear cells (PBMC) stimulated with the antigen by the CPM of the corresponding control antigen-stimulated PBMC.
[b]The stimulation index (SI) was derived from the CPM of PBMC stimulated with the antigen divided by those of the corresponding control antigen-stimulated PBMC.

response was regarded as positive if the SI was higher than 2, and the net CPM (the CPM of the antigen reduced by that of the control antigen) was more than 1,000.

Serology

Anti–HHV-6 IgG levels were determined by indirect immunofluorescence using HSB-2 cells infected with the GS strain as the antigen, and anti-CMV IgG levels were determined by ELISA according to the method published previously [Linde et al., 1990]. Plasma samples from 33 of the 36 individuals were available for analysis.

PCR Methods

The presence of HHV-6 and HHV-7 DNA in PBL was detected by PCR methods, and HHV-6 variants were determined by a variant-specific PCR [Yalcin et al., 1994; Wang et al., 1996]. The sensitivity of the PCR for HHV-6 DNA detection is 20–30 genomes for both GS and Z 29 strains and the sensitivity of the HHV-6 variant-specific PCR is about 50 genomes for both GS and Z 29 strains. The DNA of 5 × 10^4 leukocytes was used for each PCR assay. PBL from 32 of the 36 individuals were available for analysis.

Statistics

Results were analyzed by Fisher's exact test (two-tail).

RESULTS

Lymphoproliferative Response to HHV-6 GS and Z 29 Antigens

The optimal doses of the GS and Z 29 antigens for stimulation of PBMC were determined by using different concentrations of the antigens. There was a range of concentrations of HHV-6 antigens that induced lymphoproliferation (Table I). Eight μg/ml and 6 μg/ml were chosen as the optimal dose for the GS and Z 29 antigens, respectively, since the two doses induced the highest net CPM in the studied individuals.

Of all the 36 individuals, 9 of 36 (25%) had lymphoproliferative responses to the GS antigen, while 21 of 36 (58%) responded to the Z 29 antigen ($P = 0.008$). All of the nine individuals with responses to the GS antigen also responded to the Z 29 antigen. There was an individual difference in the intensity of lymphoproliferative responses induced by the GS and Z 29 antigens (Fig. 1). Four of the nine individuals had the same CPM levels to the GS antigen and the Z 29 antigen, while for the other five individuals, the CPM of the Z 29 antigen were 2–3 times greater than that of the GS antigen.

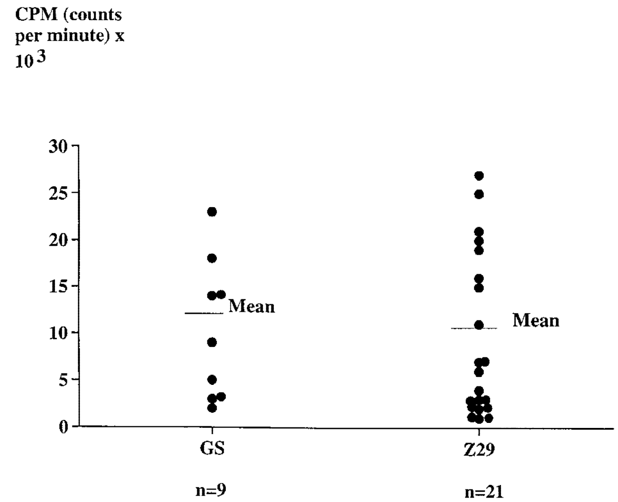

Fig. 1. Intensity of lymphoproliferative responses to the GS antigen and the Z 29 antigen. Only the counts per minute (CPM) of the individuals who had net CPM more than 1 × 10^3 and stimulation index (SI) more than 2 are shown. The net CPM and SI were obtained from reducing and dividing the CMP of the antigens by that of the corresponding control antigens.

Lymphoproliferative Responses in Control Experiments

VZV antigen, CMV antigen, and PHA were used in this study as controls of lymphoproliferation assays. Twenty of 20 (100%) individuals had lymphoproliferative responses to the VZV antigen. Thirteen of 14 (93%) CMV seropositive individuals, but none of 8 CMV seronegative individuals, had lymphoproliferative responses to the CMV antigen. All 36 individuals had lymphoproliferative responses to PHA.

The possible inhibitory effects of the HHV-6 antigens were tested on the PBMC from two individuals. No inhibition on PHA-induced lymphoproliferation was observed when the HHV-6 GS and Z 29 antigens were used at 16 μg/ml, 12 μg/ml, and 8 μg/ml, respectively.

Lymphoproliferative Responses and Serology

All 33 individuals who had plasma samples available for testing were seropositive to HHV-6, while 23 (70%) were seropositive to CMV. Eighteen of 27 (64%) individuals with low anti–HHV-6 IgG titers (≤320) had lymphoproliferative responses to the Z 29 antigen compared with 7 of 27 (30%) individuals who responded to the GS antigen ($P = 0.006$), but this difference was not found in the individuals who had high anti-HHV-6 IgG

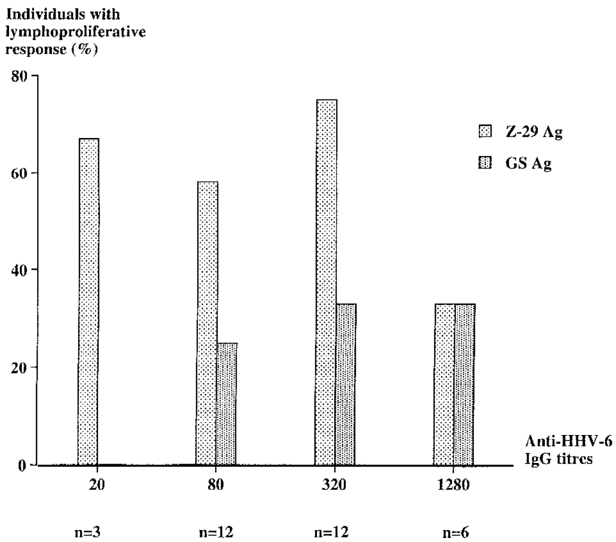

Fig. 2. Relation between lymphoproliferative responses and anti–HHV-6 IgG titers. Lymphoproliferative responses to the GS antigen were not detected in any of the three individuals with anti–HHV-6 IgG titer of 20.

titers (1,280, Fig. 2). Anti–HHV-6 IgG titers higher than 1,280 were not detected in this study. There was a tendency, although not significant, that individuals with high anti–HHV-6 IgG titers were less likely to respond to the Z 29 antigen than those with low anti–HHV-6 IgG titers. Neither CMV seropositivity nor the lymphoproliferative responses to CMV antigen were related to the lymphoproliferative responses to HHV-6 antigens (Table II).

Lymphoproliferative Responses to HHV-6 Antigens and Presence of HHV-6 and HHV-7 DNA in PBL

HHV-6 and HHV-7 DNAs were detected in the PBL of 7/32 (22%) and 18/32 (56%) individuals, respectively. Five individuals with HHV-6 DNA in PBL had variant B, while the HHV-6 of two individuals could not be subtyped. Five of 7 (71%) individuals with HHV-6 DNA in PBL had lymphoproliferative responses to the Z 29 antigen, compared with 15 of 25 (60%) individuals without detectable HHV-6 DNA in PBL (not significant, NS). The corresponding numbers for the GS antigen were 2/7 (29%) vs. 7/25 (28%), respectively (NS).

Nine of 18 (50%) individuals with detectable HHV-7 DNA in PBL had lymphoproliferative responses to the Z 29 antigen compared with 11 of 14 (78%) individuals without detectable HHV-7 DNA in PBL (NS). The corresponding numbers for the GS antigen were 5/18 (28%) vs. 4/14 (29%), respectively (NS).

Difference Between Swedish and Japanese Healthy Adults

As shown in Table III, 3 of 7 (43%) Japanese responded to the GS antigen compared with 6 of 29 (24%) Swedes (NS). Seven of 7 (100%) Japanese responded to the Z 29 antigen compared with 14 of 29 (48%) Swedes ($P = 0.03$). One of six Swedish males responded to the Z 29 antigen compared with six of six Japanese males ($P = 0.02$). The corresponding numbers for the GS antigen were 1/6 and 2/6 for the male Swedes and male Japanese, respectively ($P = 0.24$). There was also a tendency that more of the Japanese healthy adults were CMV seropositive. However, there were no differences in the lymphoproliferative responses to the CMV and VZV antigens between the Japanese and Swedish healthy adults who were seropositive to the two viruses.

DISCUSSION

In this study, glycine-extracted cellular antigens likely to contain mainly viral nucleocapsids [Kettering et al., 1977] of HHV-6 variants A and B were used in lymphoproliferation assays. The Z 29 antigen (variant B) was more efficient than the GS antigen (variant A), and induced lymphoproliferative response in 58% of HHV-6 seropositive healthy adults. As previously reported [Horvat et al., 1993], neither the GS antigen nor the Z 29 antigen at the doses used in this study inhibited lymphoproliferation induced by PHA. In addition, similar antigenicity of the antigen preparations was likely since the mean CPM of all the stimulation-positive individuals were similar for the two antigens (Fig. 1). HHV-6 variant A can induce interleukin-1β and tumor necrosis factor alpha [Flamand et al., 1991] and variant B can induce interferon alpha production in PBMC cultures [Kikuta et al., 1990]. HHV-6 may also induce cytokine production in MOLT-3 and HSB-2 cells, which might have contributed to the cell proliferation detected in this study. However, the individual variation in responses argues against a nonspecific stimulation, and it has been shown previously that lymphoproliferative responses to herpesvirus antigens are highly specific [Ljungman et al., 1985].

PCR studies have shown that most of the detected HHV-6 in healthy adults and transplant patients belonged to variant B [Di Luca et al., 1994; Singh and Carringan, 1996; Wang et al., 1996]. Although the distribution of HHV-6 variant A and B in healthy adults is not known, the results presented in this study support the hypothesis that HHV-6 variant B strains are more common than variant A strains in both Swedish and Japanese healthy adults. Yakushijin et al. [1991] reported that 13 of 14 HHV-6 seropositive Japanese healthy adults had lymphoproliferative responses to GS antigen. The virus used in their study had been propagated on PHA-stimulated cord blood mononuclear cells and was UV-treated. Our results (data not shown) indicated that a few more individuals had lymphoproliferative responses to UV-treated GS antigen than the untreated GS antigen, while UV treatment made no difference for the Z 29 antigen. However, UV treatment also increased the stimulation effects of the control antigens in some individuals (data not shown). Only UV treatment could not explain the difference of the two studies, and further analysis of T-cell memory

TABLE II. CMV Seropositivity, Lymphoproliferative Responses to CMV Antigen, and Lymphoproliferative Responses to HHV-6 Antigens

	Number of individuals (%) with lymphoproliferative responses to HHV-6 antigens		
CMV serology	GS	Z29	Total[a]
Negative	2/10 (20%)	5/10 (50%)	5/10 (50%)
Positive	7/23 (30%)	15/23 (65%)	15/23 (65%)
P value (Fisher's exact test)	1	0.46	0.46
CMV lymphoproliferation			
Negative	1/9 (11%)	4/9 (43%)	4/9 (43%)
Positive	6/13 (46%)	10/13 (77%)	10/13 (77%)
P value (Fisher's exact test)	0.16	0.19	0.19

[a]All those who had lymphoproliferative responses to the GS antigen also responded to the Z 29 antigen.

TABLE III. Differences Between Japanese and Swedish Individuals in HHV-6 and CMV Serology and in Lymphoproliferative Responses to HHV-6 Antigens

	Swedes (n = 29)	Japanese (n = 7)	*P* value (Fisher's exact test)
Age, years	39 (24–60)	33 (30–36)	
Seropositive individuals			
CMV	16/26 (62%)	7/7 (100%)	0.08
HHV-6	26/26 (100%)	7/7 (100%)	1
Individuals with Lymphoproliferative responses			
CMV antigen	6/7 (86%)[a]	7/7 (100%)	1
GS antigen	6/29 (20%)	3/7 (43%)	0.33
Z29 antigen	14/29 (48%)	7/7 (100%)	0.02

[a]Only the Swedes who were CMV seropositive were analyzed. Nine CMV seropositive Swedes were not analyzed for lymphoproliferative responses to the CMV antigen.

responses in different races in a larger population may be of interest.

It is noteworthy that some seropositive healthy adults did not have detectable lymphoproliferative responses to any of the HHV-6 antigens, despite being HHV-6 seropositive and able to respond to VZV and CMV antigens. One reason could be the nature of the antigen preparations. However, a batch of HHV-6 nuclear antigens prepared in the same way as CMV and VZV antigen was less efficient in inducing a specific response than the glycine-extracted antigen in a preliminary study (data not shown), and therefore the glycine-extracted antigen was chosen. Another reason for the difference in lymphoproliferative responses may be the nature of different viruses and their mode of infection, as well as the immune responses of the hosts. We compared anti–HHV-6 IgG titers and the lymphoproliferative responses to HHV-6 antigens. More individuals with low anti–HHV-6 IgG titers had lymphoproliferative responses to the Z 29 antigen than to the GS antigen, while there was no difference in those with high specific IgG titers. The anti–HHV-6 IgG titers were tested only with HSB-2 cells infected with the GS strain, since the results were similar when MOLT-3 cells infected with the Z 29 strain were used as the antigen [Enbom et al., 1997]. HHV-6 variant B accounts for the majority of symptomatic infections in infants [Schirmer et al., 1991; Dewhurst et al., 1993], while most of HHV-6 variant A were isolated from adults [Downing et al., 1987; Ablashi et al., 1991; Aubin et al., 1991]. It is possible that HHV-6 variant A infection occurs later in life than variant B. Although possibly asymptomatic, it may boost HHV-6–specific IgG levels and induce lymphoproliferative responses.

Specificity analysis of human $CD4^+$ T-cell clones induced by HHV-6 antigen prepared from infected cord blood mononuclear cells indicated a cross-reactivity and a close relation between HHV-6 variants A and B, and at a lower level between HHV-6/HHV-7 and CMV [Yasukawa et al., 1993]. However, the T-cell clones were derived from only a couple of individuals and it is unclear how representative of the total T-cell population reactive to the virus that the obtained clones were. Since lymphoproliferation to HHV-7 antigen was not examined in this study, cross-stimulation in the lymphoproliferation assays could not be completely excluded. However, the data suggest that HHV-7 DNA in PBL is not correlated with the lymphoproliferative responses to HHV-6 antigens. Similarly, neither CMV seropositivity nor the presence of lymphoproliferative responses to CMV antigen was related to the lymphoproliferative responses to HHV-6 antigens.

There are indications that Japanese children have clinical exanthem subitum more often than children in North America and Europe during primary HHV-6 infections [Pellett and Black, 1996]. This may suggest a difference in either the virus strains and/or in the human leukocyte antigen, thus leading to different host

responses. Japanese healthy adults were more likely to respond to the HHV-6 antigens than Swedes despite the same rates of responses to the CMV and VZV antigens. This is an indication that Swedish and Japanese healthy adults may have different rates of immune responses to HHV-6 antigens, but the results need to be confirmed in a larger population.

In conclusion, it was shown that more healthy adults have lymphoproliferative responses to the antigen from HHV-6 variant B than the antigen from HHV-6 variant A. The data suggest a higher frequency of infection with HHV-6 variant B than with variant A in the healthy population. It is possible that there is a racial difference between Caucasians and Asians in either the HHV-6 virus strains and/or the nature of host responses to HHV-6 infection. Atypical lymphoproliferative responses may occur in pathological conditions and further examination of specific lymphoproliferative responses to HHV-6 antigens may help clarify the pathological mechanisms behind HHV-6 infections.

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